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PRINCIPAL INVESTIGATOR: Andrew K. Godwin, Ph.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

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Andrew K. Godwin, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fox Chase Cancer Center
7701 Burholme Avenue
Philadelphia, Pennsylvania 19111

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We have identified a region of less than 30 kbp, on chromosome 17p13.3 by allelic loss mapping, which is altered in >50% of the breast tumors analyzed. Using positional cloning techniques we have identified several genes, one which we refer to as *OVCA2*, that fall within this critical region. To date, we have found that (1) *OVCA2* is a new gene residing in a chromosomal region which is frequently lost in breast, brain, colon, ovarian tumors, acute myeloid leukemia and myelodysplastic syndromes, (2) OVCA2 is a secretory protein which is highly evolutionarily conserved, (3) OVCA2 (25 kDa) is proteolytically processed to its mature form (21 kDa), (4) p21 is present in normal blood serum and in breast nipple aspirate fluid, (5) both forms of OVCA2 are present in a variety of tissues, including mammary epithelium, (6) secretion of OVCA2 into the breast lumen is dependent on the developmental stage of the mammary gland, and (7) secreted OVCA2 appears to be lower in most breast tumors, relative to normal mammary epithelial cells. The recent detection of $p21^{OVCA2}$ in normal serum and NAF, suggests that OVCA2 could be a new hormone with an important paracrine function. Identifying the potential role of OVCA2 in the development and the pathogenesis of the breast might ultimately help us to better understand the disease and to plan more effective treatment strategies.

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Principal Investigator's Signature

7/25/98
Date

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Fox Chase Cancer Center 07/01/96 07/31/00

Identification of Candidate Suppressor Genes on Chromosome 17p13.3: Assessment of Their Roles in Breast and Ovarian Carcinogenesis"

INTRODUCTION:

Breast cancer is the second most common form of cancer in women, striking 1 out of 8 women in their lifetime (1, 2). Ovarian cancer strikes fewer women (1, 2) but is generally at an advanced stage at the time of detection (3). Both diseases are controlled by multiple genetic defects, suggesting the involvement of many different genes, including tumor suppressors (4, 5). According to the two-hit model of Knudson (6), both alleles encoding for a tumor suppressor must be lost or inactivated in order for cancer to develop. Based on this model, loss of heterozygosity (LOH) of alleles from tumor tissue has been used to suggest the presence of potential tumor suppressor genes.

The short arm of chromosome 17 is one of the most frequently altered regions in human breast and ovarian cancer (7-11). One locus of high allelic loss is at 17p13.1, and contains the tumor suppressor gene, *TP53* (4). However, we and others have shown a second region of LOH distal to the *TP53* gene, at 17p13.3, in breast tumors (7, 9, 12-15) and ovarian tumors (16-18). Genomic abnormalities involving 17p13.3 has also been reported in primitive neuroectodermal tumors (19), carcinoma of the cervix uteri (20), medulloblastoma, osteosarcoma (21), astrocytoma (22), and acute myeloid leukemia and myleodysplastic syndromes (23) suggesting that a gene(s) on 17p13.3 may play a role in the development of a wide variety of neoplasms, including breast and ovarian cancer.

We have previously defined a minimum region of allelic loss (MRAL) on chromosome 17p13.3 in genomic DNA from ovarian tumors (17) and breast tumors (unpublished data). Positional cloning and sequencing techniques revealed two genes in the MRAL, referred to as *OVCA1* and *OVCA2*, which overlap one another in the MRAL, and have one exon in common. Since translation of *OVCA1* does not proceed into the shared exon, the genes encode for completely distinct proteins. The present study focuses on characterization of the OVCA2 protein, and demonstrates that OVCA2 is a secretory protein which is highly evolutionarily conserved, expressed in a variety of tissues, and is proteolytically processed to its mature form.

BODY:

OVCA2 is a Unique Gene which is Highly Evolutionarily Conserved.

To confirm the full-length sequence of *OVCA2*, several human cDNA libraries were screened using both conventional and PCR based procedures. In addition, several human Expressed Sequence Tags (EST's) were identified which matched the full-length cDNA of *OVCA2*. The gene contains 1010 bps, and is composed of 2 exons. Exon 2 is also exon 13 of an overlapping gene, *OVCA1* (10, 17). However, translation of *OVCA1* does not proceed into the shared exon, and OVCA2 and OVCA1 are completely distinct proteins. The full cDNA sequence of OVCA2 has recently been deposited into GenBank.

The OVCA2 protein consists of 227 amino acid protein (Figure 1). A BLAST search of GenBank/EMBL and Swissprot databases revealed that *OVCA2* does not match any known mammalian genes. However, one *C. elegans* and 4 yeast proteins were identified which showed up to 60% similarity and up to 45% identity to the amino acid sequence of OVCA2, and contained a similar number of amino acids (Figure 1). These sequences were described as putative DHFRs, but they share more conserved domains with OVCA2 than with mammalian DHFRs (data not shown). A BLAST search of the EST database revealed full-length mouse and partial rat OVCA2 homologues displaying 87% and 86% similarity, respectively, to the amino acid sequence of OVCA2. In addition, two plant ESTs (rice and arabidopsis) (up to 53% similar), and multiple human sequences were identified. A multiple sequence alignment of OVCA2 with all available non-human OVCA2 homologues (Figure 1) revealed at least 5 conserved domains, which presently have no known function, but which may be important new functional domains based on their evolutionary conservation. Zoo blots probed with the unique exon 1 of *OVCA2* demonstrated that all mammalian species tested have an *OVCA2* homologue (Figure 2a). Interestingly, when exon 2 of *OVCA2*, which is a non-coding exon of *OVCA1*, was used to probe these blots, both *OVCA2* and *OVCA1* bands were identified (Figure 2b), suggesting that the genomic arrangement of the two genes is conserved among many different species. This high degree of evolutionary conservation suggests that *OVCA2* may be very important for normal cellular function.

The Genetics Computer Group (GCG) package was used to evaluate functional motifs within the OVCA2 amino acid sequence (Figure 1). Two protein kinase C phosphorylation sites (a.a. 18 and a.a. 178), two casein kinase-2 phosphorylation sites (a.a. 76 and a.a. 84), and a possible leucine zipper variant (a.a. 95) were identified, all of which are conserved within the available mouse and rat sequences. In addition, a Myb DNA binding motif was observed (aa 83), which is identical to the native Myb motif, except for a conservative amino acid change from tryptophan to phenylalanine. Interestingly, this domain contains one of the casein kinase-2 phosphorylation sites. No

other functional groups were identified which could provide clues to the function of OVCA2.

OVCA2 has a Broad Tissue Distribution.

Multiple tissue northern blots were probed with the unique exon 1 of *OVCA2* (Figure 3a). All tissues tested showed an approximately 1.1 kb band representing the *OVCA2* transcript, with the testis showing the highest mRNA expression. This band is also detected when the shared exon 2 of OVCA2 is used as a probe (17).

An Alternative Spliced Transcript is Expressed in Testis.

We have determined the nucleotide sequence for *OVCA2* including ~16 kbp upstream of the first exon. An RNase protection assay using a probe including all of exon 1 and ~100 bases upstream of the first AUG was employed to establish the putative transcription start site. Most transcripts appeared to contain ~15 bases of 5' untranslated sequence. In testis, the transcript appeared to be slightly longer (data not shown). To evaluate the potential start site in testis, a multiple tissue northern blot was hybridized with a probe representing sequence for exons 9 through 12 of *OVCA1* (Figure 3b). No bands were detected in the size range of 1.1 kb for any of the tissues tested, except for testis mRNA. A slightly larger transcript was detected. We refined the *OVCA1* probe to include exon 12, the intron/exon boundary, and 50 bp of flanking DNA. Hybridization with exon 12 failed to detect the *OVCA2* transcripts in the testis sample. We are currently evaluating exons 9, 10, and 11 of *OVCA1* individually to help define the alternative transcript detected in testis.

OVCA2 Antibody Production.

To aid in the evaluation of OVCA2, we have generated two polyclonal antibodies (Abs) against OVCA2. A MAP peptide corresponding to a.a. 176 to 190 of OVCA2 was used for the C-terminal Ab, and a KLH-conjugated peptide (a.a. 32 to 46 of OVCA2) was used for the N-terminal Ab. Both antibodies were immunoaffinity purified prior to use. Additional antibodies (both monoclonal and polyclonal) are currently be generated to the first 35 amino acids of OVCA2.

OVCA2 Directs Expression of the Predicted 25 kDa Protein *In Vitro*.

Genomic and cDNA fragments of *OVCA2* were subcloned into the mammalian expression vectors pcDNA3 (InVitrogen). A 1,110 bp fragment was amplified by PCR using gene specific primers containing *HindIII* (5') or *EcoRI* (3') restriction endonuclease sites and genomic DNA. This fragment includes 30 bp of 5' untranslated sequence, exon 1, intron 1, and the coding sequences in exon 2. The PCR generated DNA fragments were digested and subcloned into the multiple cloning sequence of pcDNA3. To produce an N-terminal hemagglutinin (HA) tagged OVCA2 expression vector, *OVCA2* cDNA was first

cloned in frame into the *HA* containing mammalian expression vector, J3H (24), then the *HA-OVCA2* cDNA was subcloned into pcDNA3. Cos-1 cells were transfected with the genomic *OVCA2* expression vector under the control of a CMV promoter. These cells produced the predicted ~25 kDa protein, which could be detected with both a C-terminal and an N-terminal OVCA2 Ab (Figure 4). The same results were obtained when Cos-1 cells were transfected with the *HA-OVCA2* cDNA (not shown), indicating that the mRNA transcribed from the genomic DNA was correctly spliced within the cells. Untransfected MCF-7 and MDA MB-468 extracts showed a faint 25 kDa band on Western blots probed with the N-terminal Ab, demonstrating low level endogenous production of OVCA2 in cells (data not shown). However, endogenous $p25^{OVCA2}$ protein could not be detected with the C-terminal Ab, due to a much lower affinity of the C-terminal Ab than the N-terminal Ab for OVCA2.

OVCA2 is Proteolytically Cleaved in Tissues.

Western blots analysis using the N-terminal Ab, 143-3 detected a 25 kDa band in all normal tissues examined (Figure 5a). When Western blots were performed on extracts from normal human tissues using the C-terminal OVCA2 Ab, a single band was obtained in a variety of tissues, especially secretory tissues such as mammary gland, ovary, placenta and testis (Figure 5b). However, the size of the band was ~21 kDa ($p21^{OVCA2}$), rather than the predicted size of 25 kDa. Interestingly, the N-terminal Ab was not able to detect the 21 kDa protein in tissue extracts (Figure 5a). One explanation for this discrepancy is that OVCA2 is posttranslationally modified to a smaller form in tissues, but not in cells. On the other hand, it is possible that the C-terminal OVCA2 Ab cross-reacts with a protein in tissue extracts which is not OVCA2. In order to determine whether the 21 kDa protein observed in tissue extracts is actually OVCA2, mass spectral analysis was performed. The amino acid sequence of the protein in tissues is identical to the predicted amino acid sequence of OVCA2, but is missing the N-terminal portion of the protein from a.a. 1-34. Significantly, a protein cleavage site for the family of kexin-like proprotein convertases (25) is present at a.a. 34 of OVCA2. Furthermore, this cleavage site lies within the middle of the 15 a.a. peptide stretch (a.a. 27 to 41) which was used to make the N-terminal Ab, thus potentially explaining why the N-terminal Ab does not recognize the tissue form of OVCA2. Taken together, these data suggest that OVCA2 is posttranslationally processed to a smaller form in tissues, probably through proteolytic cleavage.

In order to rule out the possibility that the overexpressed and the tissue forms of OVCA2 represent splice variants, exhaustive cDNA library and marathon RACE PCR screenings were performed. No alternatively spliced transcripts (i.e., transcripts containing exon 2 of OVCA2 with an alternative exon 1), were ever obtained (except for testis), and

no alternatively spliced human OVCA2 ESTs have ever been identified in GenBank/EMBL. In addition, cells transfected with OVCA2 genomic DNA always produce the 25 kDa ($p25^{OVCA2}$) form of OVCA2 in cells, even when the genomic DNA spans 4.1 kb of sequence, and the native promoter is employed (data not shown). This suggests that the cDNA sequence of OVCA2 is correct, and reinforces the idea that a posttranslational event is responsible for the difference between exogenous and endogenous forms of OVCA2.

Liver and colon show little if any production of $p21^{OVCA2}$. In skeletal muscle and heart the OVCA2 Ab detects a 35 kDa, rather than the predicted 25 kDa protein. Studies are currently underway to elucidate the exact nature of this bigger form of OVCA2. There is not an exact correlation between tissue expression of OVCA2 message and protein (Figures 3 and 4), suggesting that OVCA2 may be a translationally regulated protein.

Alterations of *OVCA2* in cancer.

Whole cell extracts were made by incubating cells in either PBSTDS (10 mM Na_2HPO_4, 150 mM NaCl, 1% triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 0.2% NaN_3, 1 mM EDTA, 5 mM NaF, 100 µg/ml PMSF, 1µg/ml leupeptin, 0.7 µg/ml pepstatin, pH 7.25), or NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0) for 30 min. on ice, then scraping off the lysed cells and centrifuging at 180,000 x g for 30 min. All extracts were stored at -80°C. Cell and tissue extracts (25 µg of protein) were electrophoresed on a 12% SDS/polyacrylamide gel, and transferred to an Immobilon P membrane (Amersham). The membrane was probed with either the C-terminal (1:10) or the N-terminal (1:1000) anti-OVCA2 Ab, followed by horse radish peroxidase conjugated donkey anti-rabbit IgG (1:10,000), and developed using ECL reagents (Amersham).

Thirty-nine primary breast tumors have been analyzed for OVCA2 levels by Western blot analysis. Forty-one percent (16 of 39) breast tumors showed a decrease in the levels of $p21^{OVCA2}$ relative to normal breast tissue (Figure 6a). Fourteen normal/tumor breast pairs were analyzed. Forty-three percent (6 of 14) showed loss of $p21^{OVCA2}$ (Figure 6b). In comparison, $p25^{OVCA2}$ levels showed an inverse correlation. $p25^{OVCA2}$ levels were higher in tumor as compared to normal breast tissue (data not shown) and were consistent with fraction of epithelial cells present in the given tissue. Histopathological examination of the tumors showed that all were primarily epithelial, indicating that expression of the mature form of OVCA2 is dramatically reduced in breast tumors as compared to normal breast tissue. $p21^{OVCA2}$ levels were similarily reduced in ovarian tumors (39%; 23 of 59) as compared to normal ovaries (Figure 6c). Primary mammary epithelial and SV40 immortalized ovarian surface epithelial cells (HIO) failed to express detectable levels of $p21^{OVCA2}$ (Figure 7). In fact, we have failed to detect any $p21^{OVCA2}$ in any cell line tested to

date. Examination of tumors of the thyroid, pancreas, liver, bladder, kidney, lung, colon, skin, and testis were highly variable. For example, tumors of the pancreas and liver expressed significantly higher levels of $p21^{OVCA2}$ relative to their corresponding adjacent normal tissue (Figure 8a & b). Interestingly, expression of $p21^{OVCA2}$ was higher in adjacent "normal" liver diagnosed with chronic hepatitis or cirrhosis as compared to pathologically normal liver (Figure 8b, lanes 1 and 9). We are currently evaluating these protein extracts with the N-terminal antibody for $p25^{OVCA2}$ levels.

OVCA2 is a Secreted Protein.

OVCA2 is abundantly produced in a number of secretory tissues. To determine the specific cell types responsible for production, immunohistochemistry was performed on paraffin-embedded tissue sections of normal breast (Figure 9a). OVCA2 was detected in the glandular epithelial cells of the breast, as well as within secretory material in the glandular lumen, indicating that OVCA2 is made and secreted by the breast epithelium. No OVCA2 was detected in stromal or endothelial components. Western blot analysis of breast nipple aspirate fluid (NAF) from normal volunteers revealed the presence of abundant amounts of OVCA2 in the NAF, confirming that OVCA2 is secreted (Figure 8b). OVCA2 is also present in normal human serum from both men and women (Figure 8c). Significantly, the family of proprotein convertases which recognizes the putative substrate cleavage site present at a.a. 34 of OVCA2, is responsible for cleaving a vast number of prohormones to their active forms (25). We have already obtained or derived expression vectors containing *mPC1, mPC2, hPACE4, hfurin, mPC5-A, rPC7, hPAC,* and *Kex2* and will co-transfect each with *OVCA2* expression vectors and test for the presence of a cleaved product in the media.

$p21^{OVCA2}$ Levels Vary in NAFs.

Human breast nipple aspirates were obtained from consenting patients, and collection was performed as previously described (26). NAFs were collected from premenopausal women who have never been pregnant(i.e., nulliparous), premenopausal women who have been pregnant (i.e., parous), postmenopausal women, and pre- and postmenopausal women carrying a breast tumor at the time of aspiration. Ten micrograms of total protein was electrophoresed on 12% SDS-PAGE, transferred to Imobilon-P, and probed with C-terminal OVCA2 antibodies. Two distinct patterns of OVCA2 expression were observed. Pattern 1 consisted of very high levels of $p21^{OVCA2}$ while pattern 2 consisted of moderate to low levels of $p21^{OVCA2}$. In most cases there was a dramatic difference between the two patterns. As described in Table 1, 90% (9 of 10) of premenopausal parous women expressed high levels of $p21^{OVCA2}$ and 10% (1 of 10) expressed moderated levels. In comparison, none (0 of 8) of premenopausal nulliparous

expressed high levels of $p21^{OVCA2}$. Previous studies have found that the breasts of nulliparous women were predominantly composed of lobules type 1, fewer lobules type 2, with lobules type 3 almost completely absent, whereas parous women (before the age of 40) had a high frequency of lobules type 3 (27). After age 40, the number of lobules type 3 decrease, with a concomitant increase in lobules type 1 in the breast of both parous and non-parous women. We have found that 50% (3 of 6) of postmenopausal women and the majority (69%) of breast cancer bearing women expressed low levels of $p21^{OVCA2}$, suggesting that lobules type 3, which are characterized by having an average of 80 ductules or alveoli per lobule (27), are the structures that primarily secrete $p21^{OVCA2}$. We have also observed in a premenopausal parous patient, that treatment with provera (i.e., progesterone) results in decreased levels of $p21^{OVCA2}$ in the NAF. Progesterone promotes development of the lobules and alveoli of the breasts, causing alveolar cells to enlarge, and to become secretory in nature. Consistent with this observation, we do not detect $p21^{OVCA2}$ in breast milk by Western blotting, suggesting that progesterone may down regulate expression of OVCA2 or influence its maturation to the secreted form. It has been suggested that lobules type 2 are the site of origin of both lobular hyperplasia and carcinoma *in situ* and that lobules type 1 and 2 grow faster *in vitro* and have a higher DNA labeling index and a shorter doubling time than lobules type 3 (28, 29). It is conceivable that secretion of the processed form of OVCA2 is involved in early stages of breast development and differentiation, and that loss of expression/secretion of p21 may contribute to breast tumorigenesis.

In summary, *OVCA2* is a new gene identified on chromosome 17p13.3. It encodes a 227 a.a. (25 kDa) protein which is proteolytically processed to a 21 kDa secretory protein. It is produced by a number of different secretory tissues, and $p21^{OVCA2}$ is found in high levels in human serum, suggesting that it may be a new paracrine hormone. However, many more studies are necessary to demonstrate such a function. OVCA2 is highly evolutionarily conserved, suggesting that it may be important for normal cellular function. $p21^{OVCA2}$ is lower in a high percentage of breast tumors, relative to normal mammary epithelial cells. High levels of $p21^{OVCA2}$ are detected in NAF of parous premenopausal women as compared to nulliparous and breast tumor bearing women. Thus, the inability to proteolytically process the proprotein may be very important in the pathogenesis of the breast. Based on these observations, it is critical that we continue to characterize the normal function of OVCA2 and determine the biological consequences of decreased $p21^{OVCA2}$ expression with respect to breast cancer development. Specifically, it is essential that we investigate OVCA2's tumor suppressive activity, its biochemical

mechanism of action, and identify the proteins that postranslationally modify $p25^{OVCA2}$ to its mature form.

CONCLUSIONS:

In order for future therapies to be developed for the fight against cancer it is important to understand the basic molecular mechanisms that give rise to a specific cancer type. The fundamental mechanisms underlying the genetic basis of cancer are slowly being defined and involve alterations in genes which have been classified into three general categories: (i) protooncogenes are involved in growth promotion and the defects leading to cancer are a gain of function; (ii) tumor suppressor genes are negative regulators of growth and a loss of function gives rise to cancer; and (iii) DNA repair genes are involved in maintaining the fidelity of the genome and altered function can lead to increase rates of mutations in both classes of cancer-causing genes. Cancer is a multistep process that involves alterations in many specific genes. The normal cell has multiple independent mechanisms that regulate its growth and differentiation and several separate events are required to override these control mechanisms. Progress is now being made in isolating these genes and the proteins they encode for, determining the normal cellular functions of the proteins and in investigating the mechanisms of tumorigenesis.

Breast cancer is a very common disease, causing about 10% of deaths in women in the Western World (30). Molecular genetic analysis of breast tumors has revealed many genetic aberrations that may represent important steps in tumor development. To understand the genetic pathways underlying breast tumor development, it is necessary to identify the genes affected by these genetic aberrations and establish any correlations between disruption of their function and tumor phenotype.

Chromosome 17 frequently shows loss of heterozygosity (LOH) in breast carcinomas (13-15, 19, 21, 31). In addition, re-introduction of chromosome 17 fragments into breast cancer cell lines has been shown to suppress tumorigenicity (32). Therefore, inactivation of tumor suppressor genes on chromosome 17 appears to be a critical event in the pathogenesis of breast cancer. Although *TP53* at chromosome 17p13.1 is involved in the pathogenesis of breast cancer, LOH mapping studies in breast, ovarian and brain carcinomas have defined a region distal to *TP53*, at 17p13.3, thought to harbor a tumor suppressor gene (13-15, 19, 21, 31). In addition, a fragment containing 17p13.3 has been shown to suppress the tumorigenicity of breast cancer cell lines (33). A new gene, *OVCA2*, has been identified on chromosome 17p13.3, in this critical region of allelic loss. *OVCA2* is composed of two exons: a unique exon 1, and an exon 2 which comprises part

of the 3' untranslated region of *OVCA1*. Thus, the two genes are overlapping, but their protein products are completely distinct.

We have found that OVCA2 is a secretory protein which is abundantly produced by secretory tissues such as breast, ovary, testis, placenta and stomach. It is actively secreted into nipple aspirate fluid, and is present in the serum of men and women. Most peptide hormones are synthesized as prohomones, which must be proteloytically cleaved to their mature, active forms (25, 34). The same appears to be true for OVCA2, which is initially translated as a 25 kDa protein, but is processed to a 21 kDa form in tissues. The mass spectral data showing an absence of the first 34 N-terminal a.a.'s in the 25 kDa form of the protein is consistent with the presence, at a.a. 30-34 of OVCA2, of a cleavage site for the kexin-like family of proprotein convertases of the general form (R/K)-Xn-(R/K) (single aa symbols; n=0,2,4,6) (25).

Although it has been known for many years that a vast array of prohormones, growth factors and neurotransmitters are cleaved at single and pairs of basic amino acids (25), the proprotein convertases (PC's) responsible for such cleavage have only been identified over the past decade (35). Thus far, seven mammalian PC family members have been identified. These include furin, PC1/PC3, PC2, PC4, PC5/PC6 (a&b), PACE4, and PC7, which differ from one another in tissue distribution, subcellular localization, and substrate specificity. Of those enzymes that recognize a 4 a.a. cleavage site, such as furin, most require arginine rather than lysine in the P4 position (R_{P4}-X_{P3}-X_{P2}-R_{P1}). The potential cleavage site of OVCA2 (K-X-X-R), although a less common variant, is identical to the proIGF-I PC cleavage site required to produce the fully mature IGF-I (36). Duguay and coworkers have demonstrated that proIGF-I can be processed by furin, PC6A and LPC (PC7) (37), so it is likely that OVCA2 can be cleaved by these enzymes, as well. In addition, the pair of basic amino acids, R-K, at the P5 and P4 positions of the above motif in OVCA2 could act as a substrate for PC's such as PC1 and PC2. Current investigations are focused on determining which enzyme(s) might play a role in OVCA2 processing (see below).

The fact that the mature 21 kDa form of OVCA2 has not been detected in cell lines is consistent with a characteristically low expression of PC's in cultured cells. (25). This phenomenon has hampered the study of mature peptide hormones *in vitro*, but researchers are now able to study cleavage by co-transfecting cells with both their gene of interest and the appropriate PC gene (38). This approach has allowed the successful *in vitro* processing of von Willebrand factor (39), and a number of other hormones, and we are currently taking this approach to identify the particular PC responsible for cleavage of OVCA2. Determining the OVCA2-specific PC may provide further insights into the nature of

OVCA2 secretion, since constituitively secreted factors are cleaved by different PC's than those whose secretion is regulated (25).

Another aspect of OVCA2 production in cultured cells is that although the breast cancer cell lines MCF-7 and MDA MB-468 cells express the full-length 25 kDa OVCA2, it is much less abundant in cell extracts than the 21 kDa form is in normal mammary tissue. Therefore, not only is there an absence of OVCA2 cleavage in the cells but also reduced protein production. It may be that the cells require a three dimensional arrangement and a columnar morphology in order to actively produce OVCA2. Or, perhaps OVCA2 production is turned off in actively growing cells. This is an interesting possiblity given the fact that OVCA2 resides in a chromosomal region which is frequently lost in many types of cancers, and is the reason it is currently being investigated as a putative tumor suppressor gene. OVCA2 expression may be under translational or posttranslational control rather than under transcriptional regulation, given the lack of correlation between protein and mRNA levels in both cultured cells and normal human tissues. In that case, there may be differences in stability between the full-length and the processed forms of OVCA2, and OVCA2 levels may depend on the ability of the tissue or cell to effectively cleave OVCA2 to an active form.

It is not clear what becomes of the 34 a.a. peptide which is cleavexd from the N-terminal of OVCA2 and is the reason we are generating both mono- and polyclonal antibodies to this peptide. The N-terminal portion of the protein differs greatly from the mature 21 kDa portion of the protein in charge and isoelectric focusing point (pH 12 vs. pH 4.1). It may be simply degraded, or as in the case of proopiomelanocortin, which is cleaved into three different active peptides, it may have its own important physiological role. This possibility is supported by the high degree of conservation of the N-terminal region of OVCA2 among all OVCA2 homologues. Interestingly, we have been unable to express the two portions of the protein separately in cells, indicating that full-length translation and posttranslational processing is necessary to generate a stable OVCA2 protein, and suggesting that another processing step in conjunction with cleavage may be operating to enhance stability of the protein.

We are not yet certain of the source of serum OVCA2. The breast produces OVCA2 and secretes it into the NAF, but this is probably not sufficient to achieve the substantial serum levels observed. Furthermore, we have not observed appreciable differences between male and female serum levels of the protein. Since we have also cloned the mouse *OVCA2* gene, perhaps we can use a mouse model to further elucidate the major origin of OVCA2 *in vivo*.

Overall, we have addressed portions of all three **Specific Aims** during the current year and have initiated several new projects all directed towards furthering our understanding of the role of OVCA2 in the development of cancer. In this aspect, we will 1) investigate OVCA2's tumor suppressive activity, 2) determine its biochemical mechanism of action, 3) identify the proteins that postranslationally modify $p25^{OVCA2}$, 4) continue to raise antibodies to OVCA2 that can routinely be used for immunohistochemistry and immunoprecipitations, and 5) investigate the biochemical mechanism of action of the OVCA2 protein by studying OVCA2 protein interactors and the effects of disrupting these normal interactions *in vivo*. In summary, we feel that the recent discoveries presented above and the studies proposed will continue to enhance our understanding of the molecular genetic events involved in the development of breast cancer, as well as potentially other neoplasias and genetic disorders involving chromosome 17p13.3.

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TABLE 1 Influence of Parity and Cancer Status on $p21^{OVCA2}$ Levels in Nipple Aspirate Fluids

Sample	$p21^{OVCA2}$ Levels	
	High	**Moderate/Low**
Premenopausal (n=24)	50%	50%
Parous (n=10)	90%	10%
Parous w/Breast tumor (n=5)	60%	40%
Nulliparous (n=8)	0%	100%
Nulliparous w/Breast tumor (n=1)	0%	100%
Postmenopausal (n=15)	33%	67%
Parous/Nulliparous (n=6)	50%	50%
With Breast tumor (n=9)	22%	78%
Pre- and Postmenapausal (n=39)	44%	56%
With Breast Tumor (n=15)	33%	67%
Without Tumor (n=24)	50%	50%

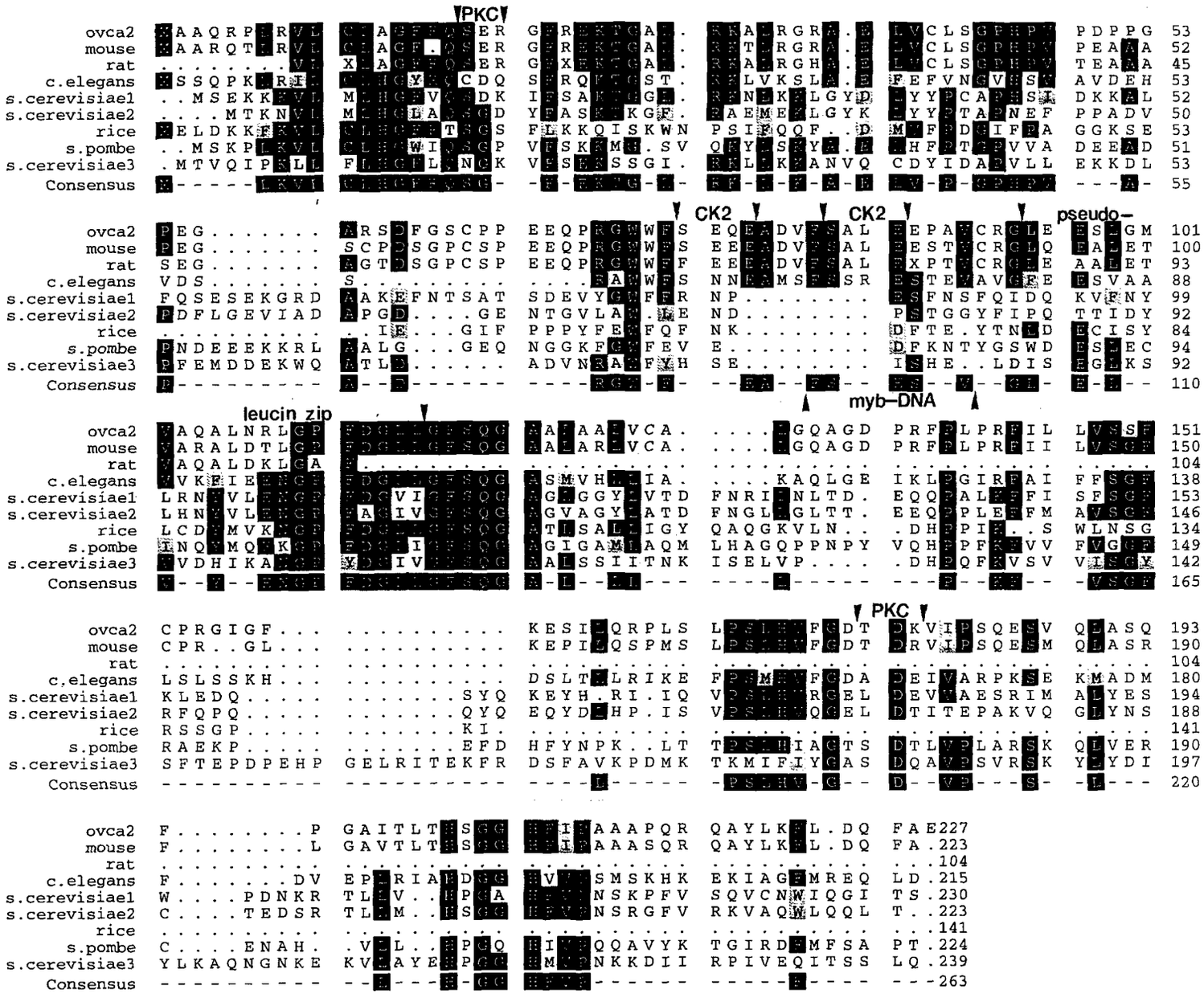


Figure 1. Evolutionary conservation of OVCA2. Multiple sequence alignment of OVCA2 amino acid sequence and similar sequences from mouse, rat, nematode, yeast, rice, and arabidopsis. At least 5 conserved regions are evident. Two protein kinase C phosphorylation site (PKC), two casein kinase-2 sites (CK2), a potential pseudo-leucine zipper motif (p-leu), and a potential myb DNA binding site (myb-DNA) are all indicated.

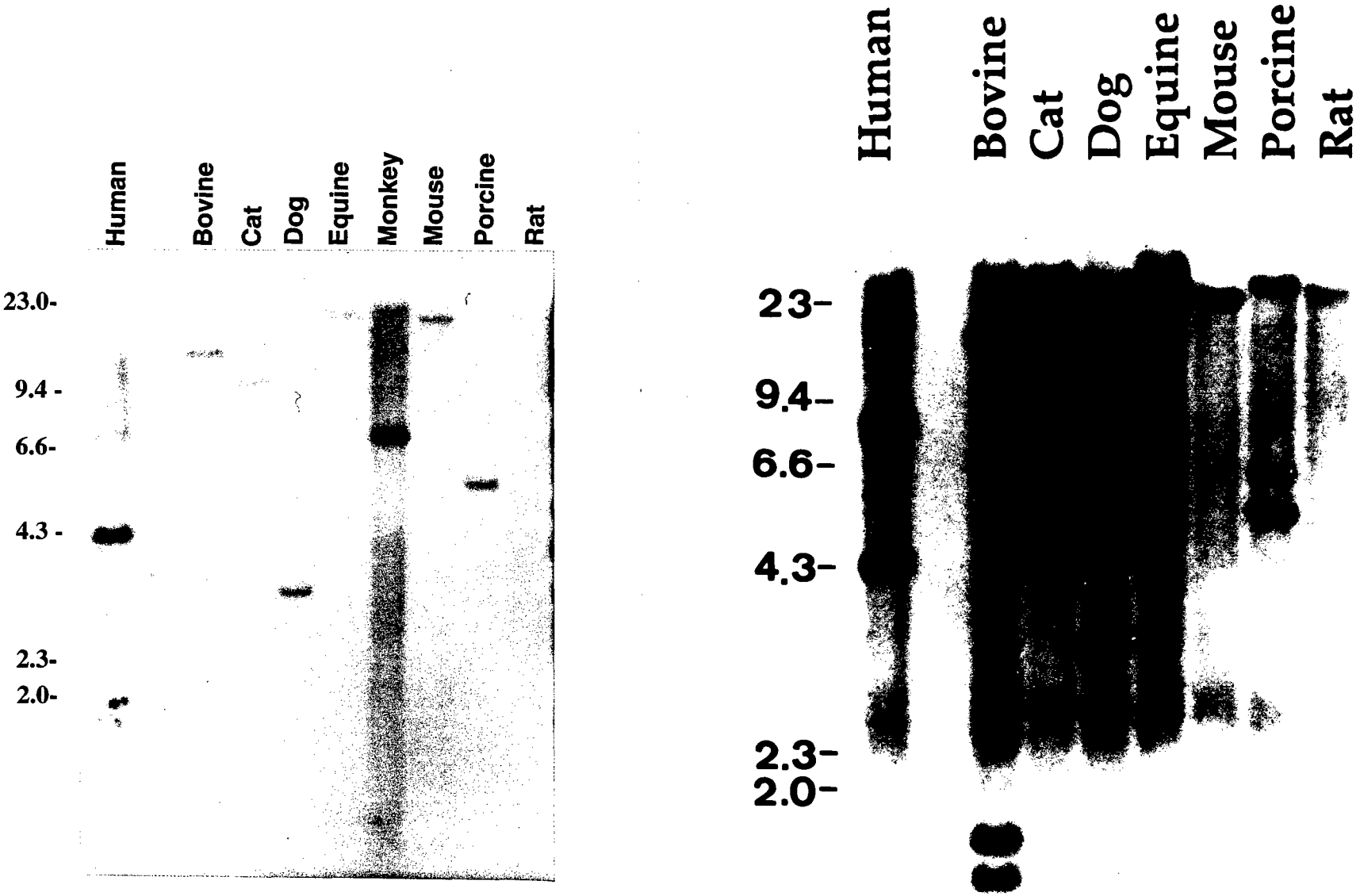


Figure 2. Genomic organization of the *OVCA1* and *OVCA2* genes are conserved. Southern blot analysis showing cross-species hybridization of a) a 185 bp *OVCA2* exon 1 probe (not shared with *OVCA1*) and b) a 2.0 kbp *OVCA1* cDNA probe to *EcoRI* genomic DNA restriction fragments from various species. Size standards are in kilobase pairs.

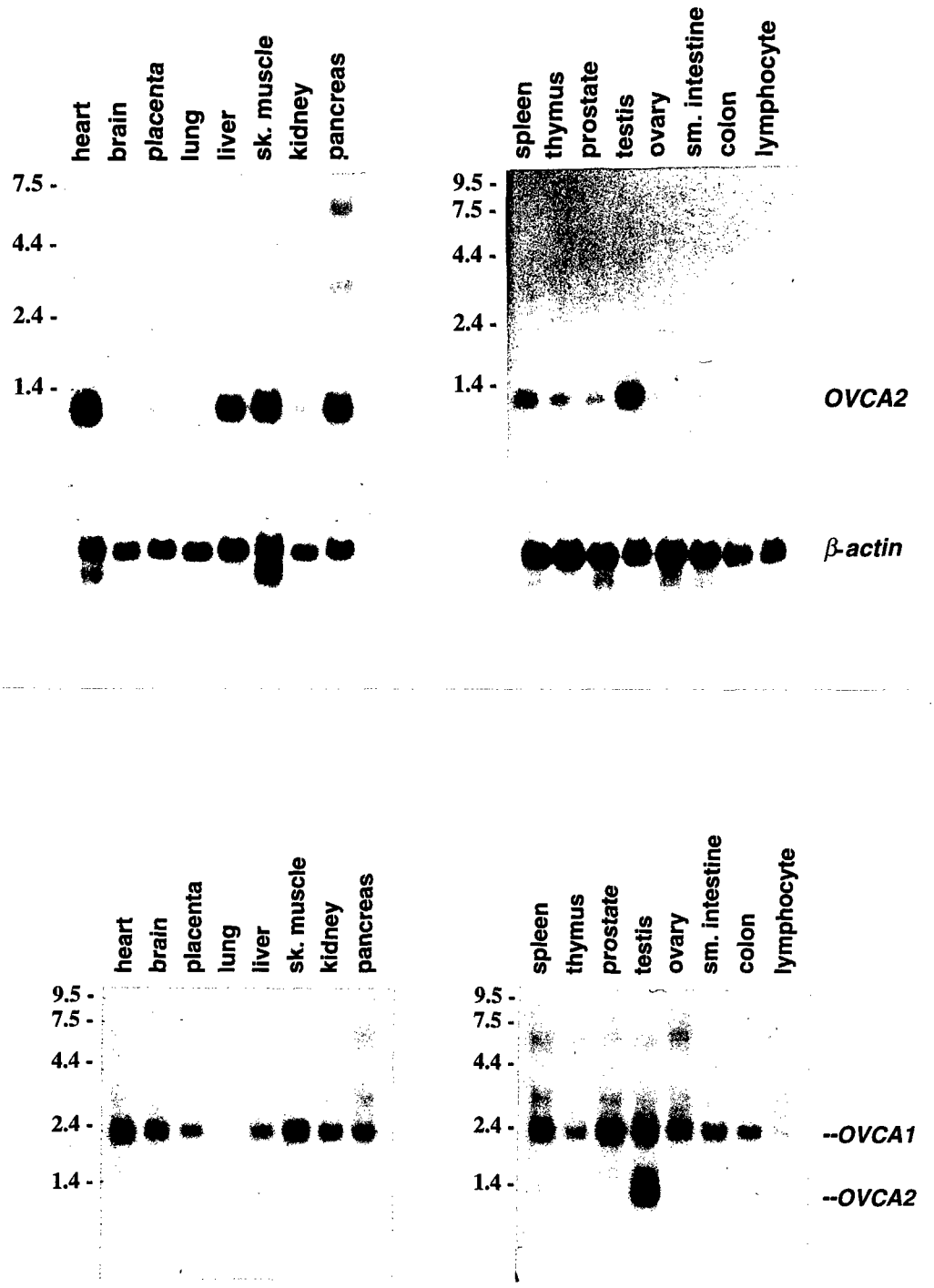


Figure 3. Detection of *OVCA2* mRNA in various tissues. Blots containing 5 μg of polyA+ selected mRNA from human tissues were hybridized with a cDNA probe corresponding to a) exon 1 of *OVCA2* (upper panel) or b) exons 9-12 of *OVCA1* (lower panel).

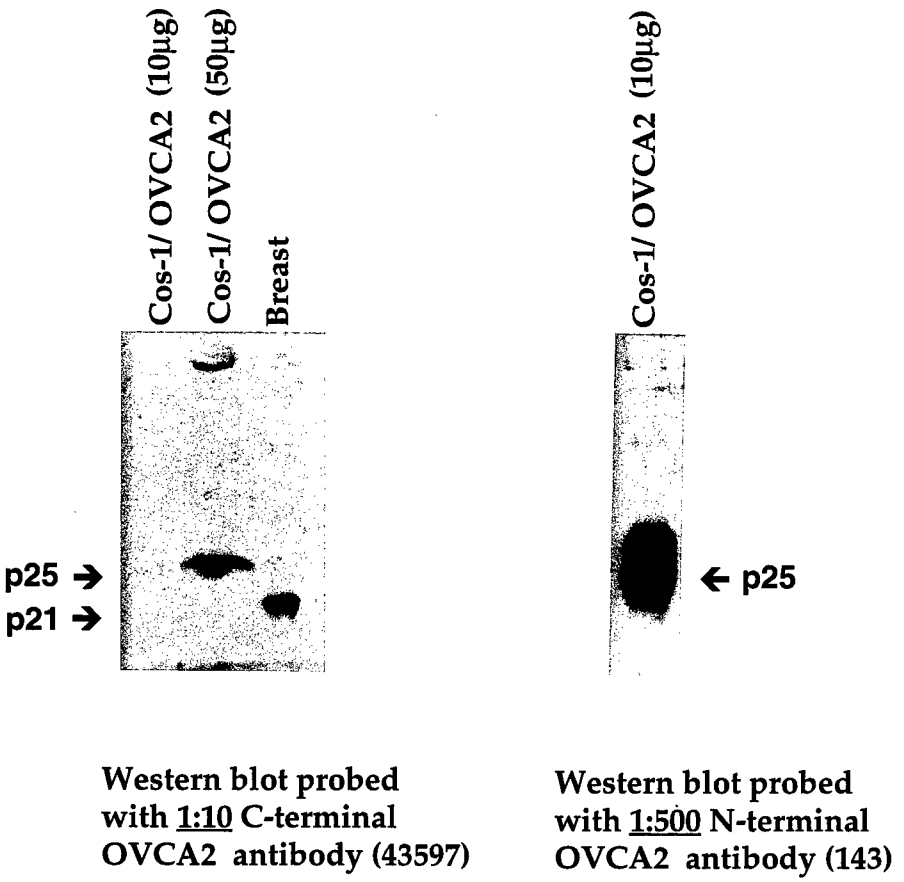


Figure 4. Evaluation of polyclonal antibodies raised against OVCA2. Detection of transiently expressed OVCA2 in COS cells extracts with affinity-purified rabbit polyclonal antiserum. COS cells were transfected with a OVCA2 expression vector and the extracts were isolated 48-72 hours later. Left panel: C-terminal (a.a. 176-190) antibody, 43597, used at a 1:10 dilution. Right Panel: N-terminal (a.a. 32-46) antibody, 143-3, used at a 1:500 dilution.

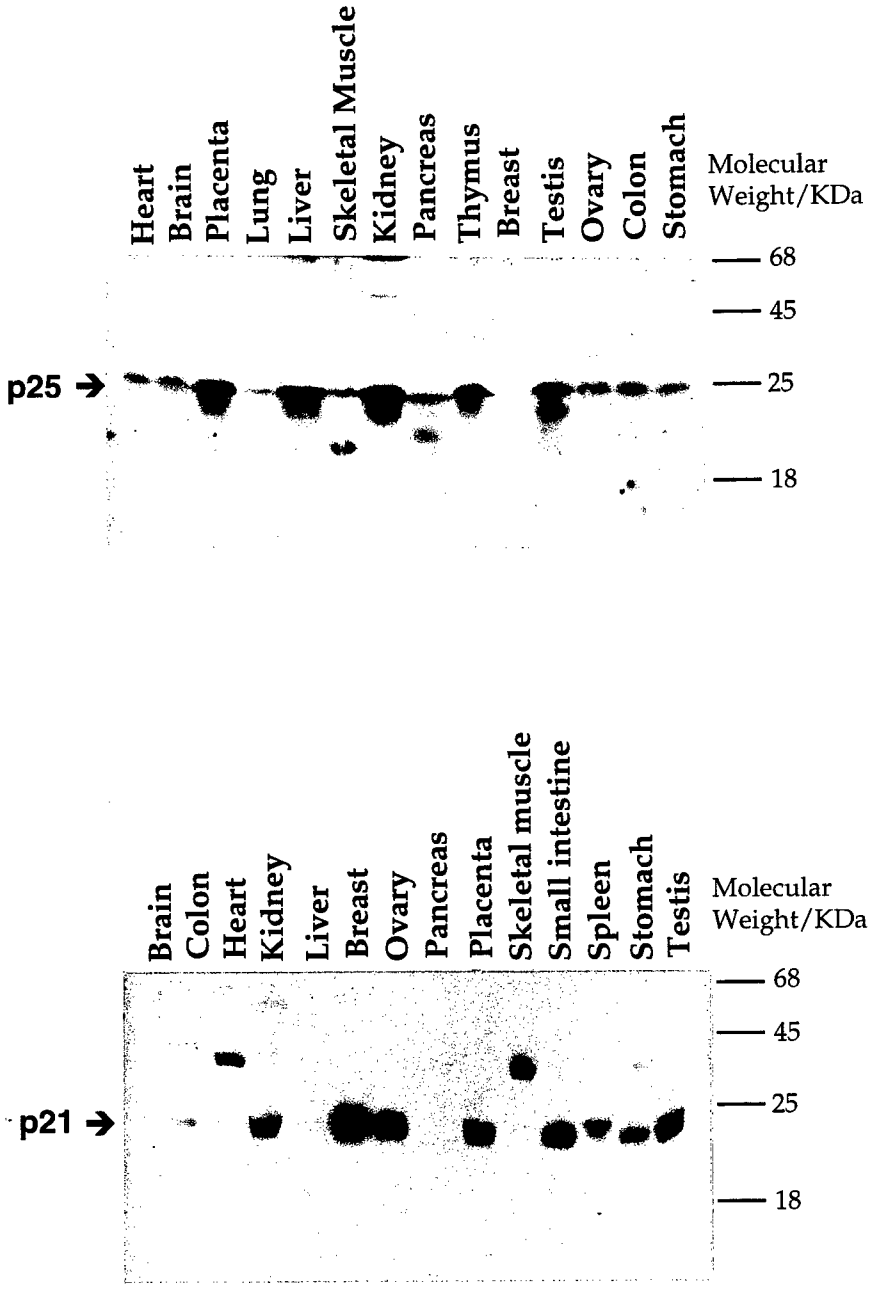


Figure 5. Multiple Tissue Western Blot. Fifty micrograms of total protein from the indicated human tissues were separated by SDS-PAGE and transferred to PVDF membrane. A) $p25^{OVCA2}$ was detected with the N-terminal, 143-3 polyclonal Ab (upper panel). Protein levels were highest in placenta, liver, kidney, thymus, and testis. B) $p21^{OVCA2}$ was detected with the C-terminal, 43579 polyclonal Ab (lower panel). Most tissues tested express a smaller than expect protein (~21 kDa versus 25 kDa). A 32-35 kDa protein is present in heart and skeletal muscle

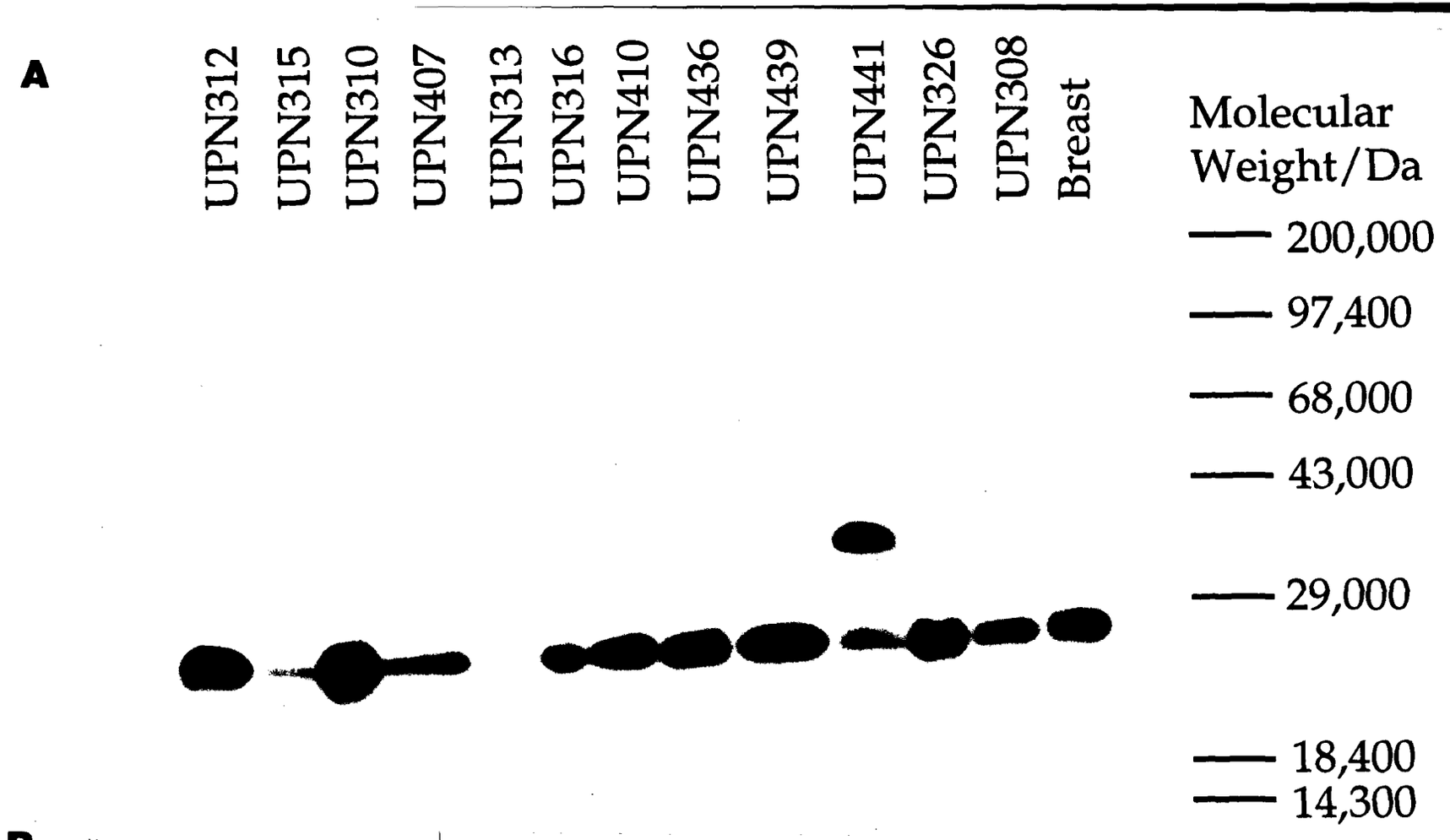


Figure 6. p21^{OVCA2} levels in breast and ovarian tumors. A) Expression of OVCA2 in breast tumor, B) breast normal/tumor pairs, and C) ovarian tumor extracts. Protein was detected using the C-terminal OVCA2 polyclonal anti-serum. Note that in UPN 441, OVCA2 migrates as a larger species. Markers shown in this western gel and subsequent gels were prestained. Note: The 29 kDa prestained marker migrates with a 25 kDa unstained marker.

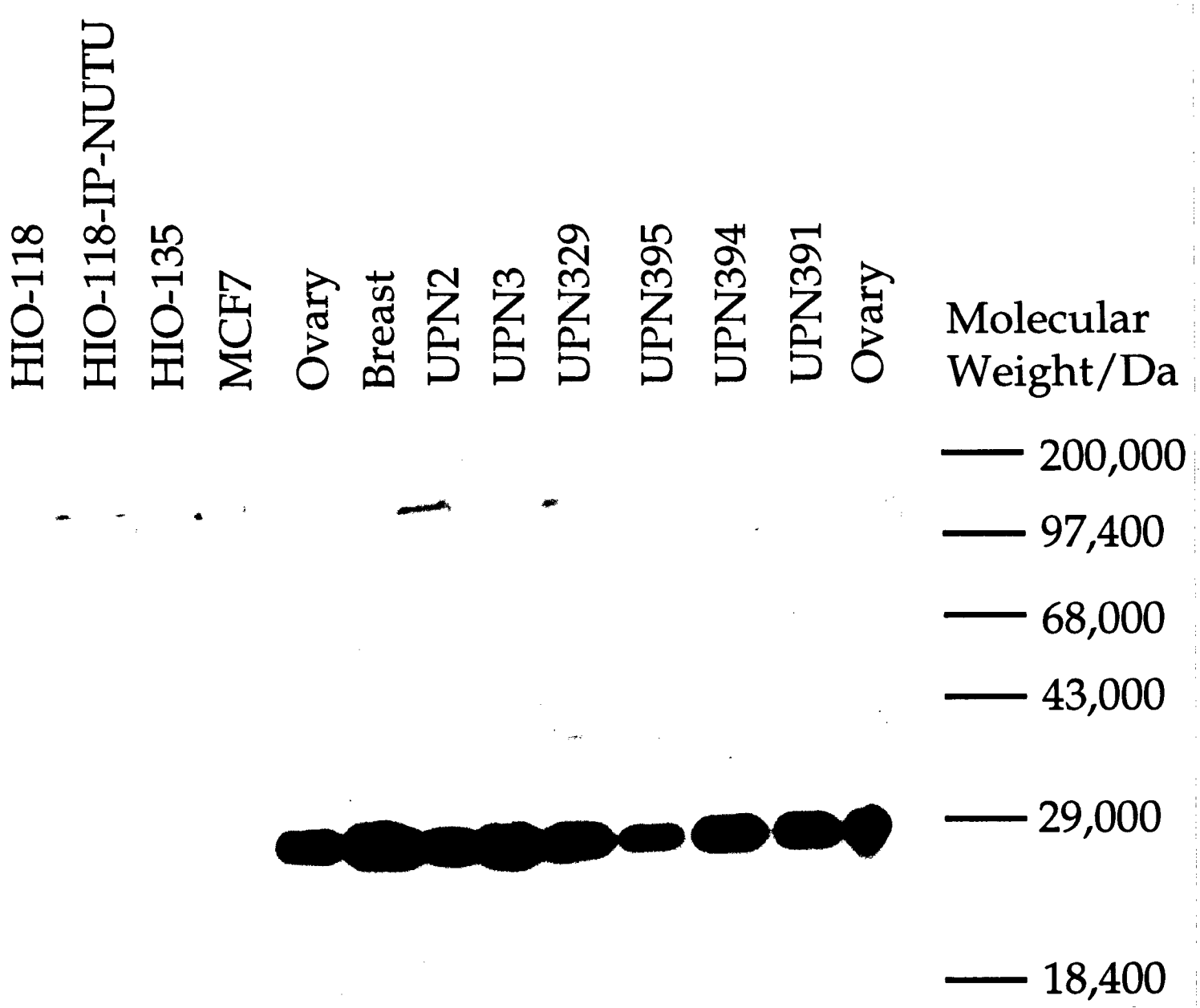
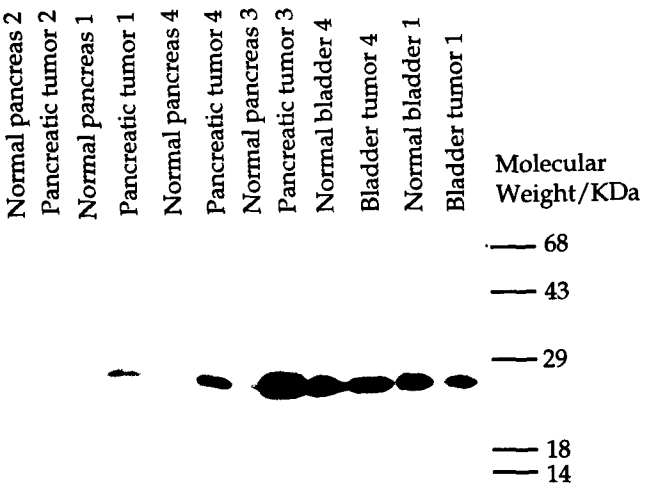


Figure 7. p21^{OVCA2} is not expressed in cell lines. Expression of OVCA2 in normal human ovary and breast extracts, immortal HOSE cells (HIO-118, 118-IP-NuTu, 135), breast tumor cell line (MCF7), and protein extracts prepared from ovarian (UPN 2 and 3) and breast (UPN 329, 395, 394, 391) tumors. Protein was detected using the anti-OVCA2 (C-terminal) polyclonal anti-serum. Note that p21^{OVCA2} is not detected in the HOSE and MCF7 cell lines.



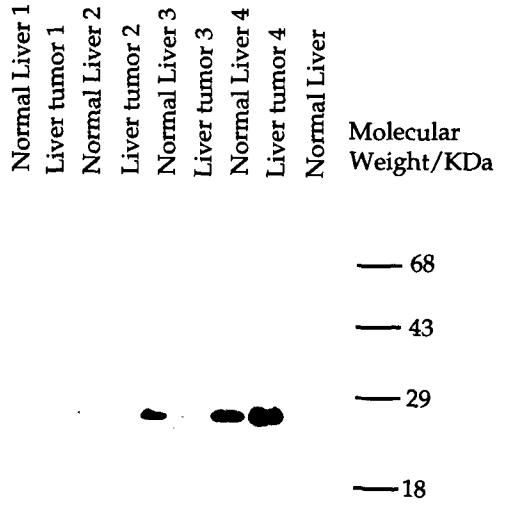


Figure 8. Western blot analysis of p21^{OVCA2} in tumors of the pancreas, bladder, and liver. Comparison of p21^{OVCA2} levels A) in normal/tumor pancreatic pairs and normal/tumor bladder pairs pancreas, and B) in normal/tumor liver pairs. Protein was detected as described in figure legend 6.

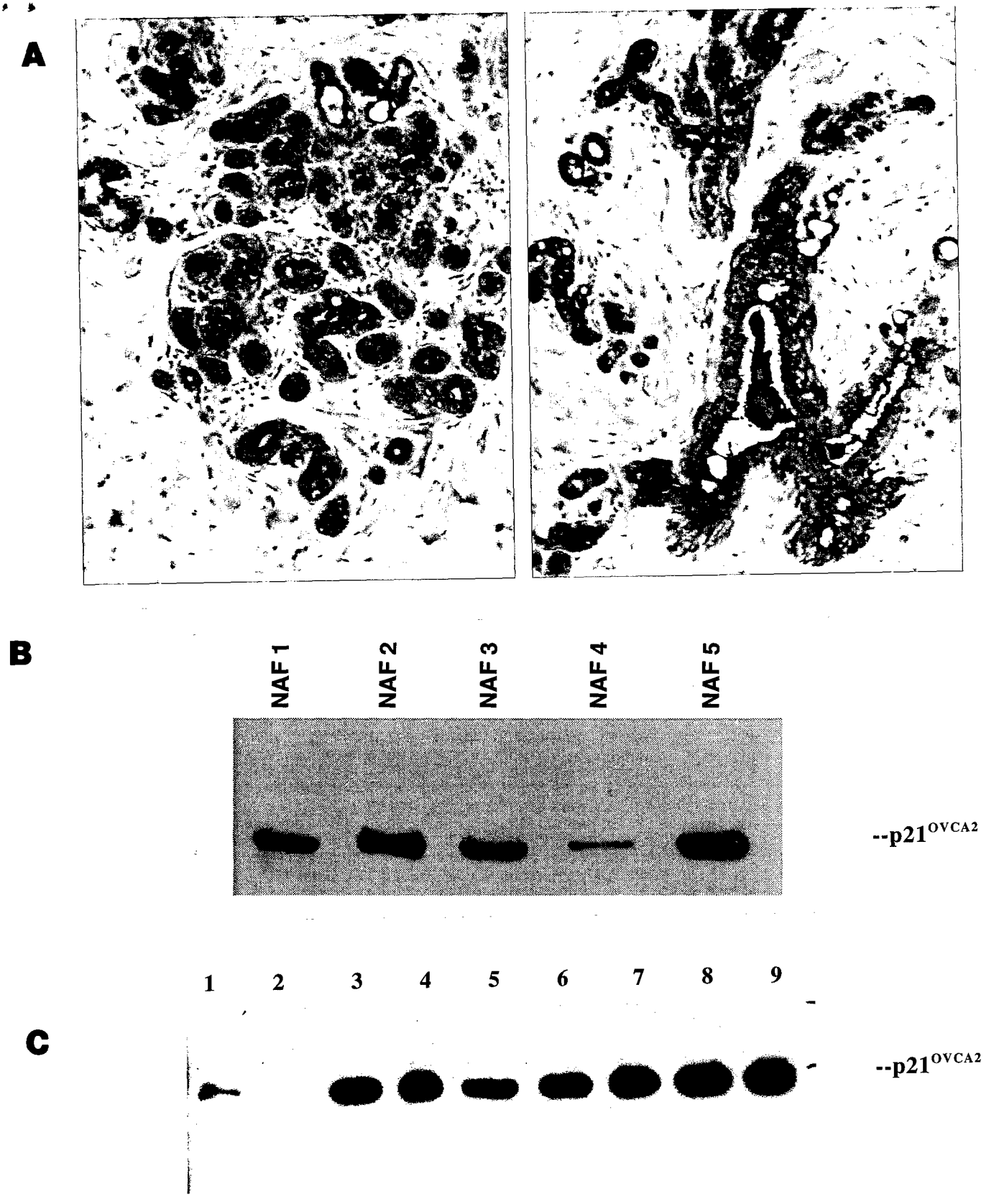


Figure 9. OVCA2 is a secreted protein. A) Immunohistochemistry of paraffin embedded normal breast tissue using 1:100 anti-OVCA2 Ab, 143-3. Glandular epithelium and secreted material within glandular lumen are positive for OVCA2. B) Western blot analysis of nipple aspirate fluid (NAF) from 5 normal women. Ten micrograms (NAF 1-3, 5) or 8μg (NAF4) were loaded per lane, and protein filters probed with 1:10 anti-OVCA2 C-terminal AB, 43597. C) Western blot analysis of blood plasma from normal women and women. Westerns were performed using the 43597 antibody as described in B. Lane 1, normal breast, 2, normal ovary, 3-9, blood plasma.